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Alpha/Beta Interferons Regulate Murine Gammaherpesvirus Latent Gene Expression and Reactivation from Latency

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Alpha/beta interferon (IFN-α/β) protects the host from virus infection by inhibition of lytic virus replication in infected cells and modulation of the antiviral cell-mediated immune response. To determine whether IFN-α/β also modulates the virus-host interaction during latent virus infection, we infected mice lacking the IFN-α/β receptor (IFN-α/βR$^{-/-}$) and wild-type (wt; 129S2/SvPas) mice with murine gammaherpesvirus 68 (γHV68), a lymphotropic gamma-2-herpesvirus that establishes latent infection in B cells, macrophages, and dendritic cells. IFN-α/βR$^{-/-}$ mice cleared low-dose intranasal γHV68 infection with wt kinetics and harbored essentially wt frequencies of latently infected cells in both peritoneum and spleen by 28 days postinfection. However, latent virus in peritoneal cells and splenocytes from IFN-α/βR$^{-/-}$ mice reactivated ex vivo with >40-fold- and 5-fold-enhanced efficiency, respectively, compared to wt cells. Depletion of IFN-α/β from wt mice during viral latency also significantly increased viral reactivation, demonstrating an antiviral function of IFN-α/β during latency. Viral reactivation efficiency was temporally regulated in both wt and IFN-α/βR$^{-/-}$ mice. The mechanism of IFN-α/βR action was distinct from that of IFN-γR, since IFN-α/βR$^{-/-}$ mice did not display persistent virus replication in vivo. Analysis of viral latent gene expression in vivo demonstrated specific upregulation of the latency-associated gene M2, which is required for efficient reactivation from latency, in IFN-α/βR$^{-/-}$ splenocytes. These data demonstrate that an IFN-α/β-induced pathway regulates γHV68 gene expression patterns during latent viral infection in vivo and that IFN-α/β plays a critical role in inhibiting viral reactivation during latency.

Alpha/beta interferon (IFN-α/β) is a critical component of the innate immune response to viral infection and exerts its protective effects both by direct inhibition of viral replication and by indirect effects on the activation of the cellular immune response (75). Direct antiviral effects of IFN-α/β include induction of apoptosis and inhibition of viral protein synthesis by PKR (93), RNase L-mediated degradation of viral mRNA (64), and inhibition of viral RNA expression by the Mx family of genes (48). However, mice lacking all of these well-characterized pathways retain significant IFN-α/β-dependent antiviral responses, demonstrating that IFN-α/β induces additional effector molecules that inhibit viral replication (42, 67, 100). One such novel IFN-α/β-induced molecule is the ubiquitin-like protein ISG15, which has now been demonstrated to have an antiviral role against Sindbis virus infection in vivo (47a). It is likely that many IFN-α/β-induced antiviral effector mechanisms will be tissue and virus specific, underscoring the importance of using diverse viral models to study these innate antiviral cytokines (18, 42, 68, 99).

IFN-α/β is expressed in large amounts early during viral infection by plasmacytoid dendritic cells, also known as interferon-producing cells (11). IFN-α/β induction during viral infection not only serves to curtail viral replication but also provides important activation and differentiation signals for the developing adaptive immune response (6). NK cells display enhanced cytolytic and proliferative capacity following IFN-α/β exposure, which may be important in the elimination of virus-infected cells early during infection (7). The capacity of IFN-α/β to upregulate the class I major histocompatibility complex antigen presentation pathway on most somatic cells is well established (75). Professional antigen-presenting cells, including immature dendritic cells, respond to IFN-α/β by upregulating several molecules important for presentation of antigenic peptides to T cells in an activating manner (23, 45, 69). The presence of IFN-α/β during initiation of T-cell activation promotes differentiation of a Th1-cell-mediated response and enhances proliferation, cytolysis, and maintenance of CD8$^+$ cytotoxic T lymphocytes (CTL) (1, 52, 60). Because of these functions provided by IFN-α/β, the production of these cytokines by interferon-producing cells is considered to be an important bridge between the innate and adaptive responses to intracellular pathogens (19).

Most of the well-characterized direct and indirect effects of IFN-α/β action during viral infection, however, have been studied primarily during the earliest stages of virus infection, prior to the maturation of the antigen-specific B- and T-cell-mediated response (72). Furthermore, the most detailed studies of IFN-α/β action have often utilized small RNA-genome viruses that typically establish only brief lytic infection in immunocompetent mice (5, 26, 33, 59, 82). While these studies have illuminated the critical role of IFN-α/β in the response to acute virus infection, they do not address possible roles for IFN-α/β during lifelong viral persistence in a latent, predom-

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inantly nonreplicating state. The herpesviruses, a significant cause of human morbidity due to their capacity for lifelong infection, periodic reactivation, and association with tumorigenesis, also require IFN-α/β to limit acute infection (14, 24, 32, 47), but the role of these cytokines in regulating latent infection and reactivation has not been carefully examined. Furthermore, the human herpesviruses carry genes that inhibit IFN-α/β action (46, 55, 58), and a subset of these genes is expressed during viral latency (9, 31, 65, 102), suggesting that IFN-α/β continues to exert antiviral effects after latency is established. This hypothesis has been difficult to test in the absence of small animal models for the human gammaherpesviruses, although in vitro evidence suggests that IFN-α/β can alter viral latent gene expression and reactivation from latency (12, 57) and IFN-α/β can inhibit the growth of lymphomas latently infected with Kaposi's sarcoma-associated herpesvirus (KSHV; also called human herpesvirus 8 [HHV-8]) and adoptively transferred into immunocompromised mice (94).

To define the physiologic effects of IFN-α/β during latent virus infection, we infected mice lacking IFN-α/β responsiveness with murine gammaherpesvirus 68 (γHV68) and measured viral gene expression and reactivation during latent infection. γHV68 is a member of the gamma-2-herpesvirus family, is closely related to Epstein-Barr virus (EBV or HHV-4) and KSHV, establishes lifelong latent infection in murine B lymphocytes, macrophages, and dendritic cells (74, 85, 87), and induces lymphoproliferative disease and lymphoma in immunocompromised mice (78a). IFN-α/β is critical for controlling acute γHV68 infection but has not been reported to regulate latent γHV68 infection (3, 24, 90). We find that IFN-α/β is not required for complete clearance of lytic γHV68 infection or for establishment of normal levels of latent virus in spleen and peritoneal exudate cells. However, latently infected peritoneal cells and splenocytes that lack IFN-α/β responsiveness demonstrate specific alterations in latent viral gene expression and profoundly increased efficiency of reactivation from latency. These findings indicate that IFN-α/β is required to direct gammaherpesvirus infection into a quiescent state of latency and demonstrate that IFN-α/β-induced signaling continues to regulate viral gene expression patterns after detectable lytic replication has ceased, illuminating a previously unappreciated antiviral function for IFN-α/β during latent virus infection.

MATERIALS AND METHODS

Viruses, mice, and infections. Murine γHV68 strain WUMS is a laboratory stock and was prepared as a third or fourth passage in murine 3T12 fibroblasts. Infectious titer was determined by plaque assay on 3T12 monolayers as described previously (89). Mice were housed by the Washington University Department of Comparative Medicine in accordance with all applicable federal standards. Wild-type (wt) mice (129S2/SvPas) (22) and mice lacking the alpha/beta interferon receptor chain 1 (IFN-α/β$R^{-/-}$), the gamma interferon receptor chain 1 (IFN-γ$R^{-/-}$), or both receptors (IFN-α/βγ$R^{-/-}$) were obtained from B & K Universal (Hull, England, United Kingdom) (82). Mice lacking STAT1 were derived as described previously (56). Eight- to 12-week-old mice were anesthetized via metofane or halothane inhalation and infected with 100 PFU of γHV68 in a volume of 40 μl complete 3T12 culture medium (Dulbecco's modified Eagle's medium [MEM], 10% fetal bovine serum) via intranasal aspiration.

Assays for latency. On the indicated day postinfection, the frequency of virus genome-positive cells and the capacity of these cells to reactivate lytic γHV68 replication were determined as previously described (89, 91). Briefly, mice were euthanized by halothane inhalation and peritoneal exudate cells were removed by peritoneal lavage with 10 ml of ice-cold complete culture medium. Spleens were homogenized to single-cell suspensions, erythrocytes were hypotonically lysed, and cell viability and concentration were determined by trypan blue exclusion. Cell preparations were serially diluted and plated immediately for the purposes of assessing viral reactivation or were cryopreserved in 10% (vol/vol) dimethyl sulfoxide at −80°C prior to limiting dilution PCR for viral genome.

To determine the frequency of explanted cells that harbored viral genome (and are therefore presumed to be latently infected [74, 87]), cryopreserved cells were thawed, counted, and serially diluted in 96-well thermal cycling plates. Cells were lysed by overnight incubation with proteinase K. Nested PCR was performed using primers specific for γHV68 ORF72 (54). Control reactions performed in the same plates verified that the nested PCR was capable of detecting a single copy of viral DNA per well in 30% of reaction mixtures and 10 copies of viral DNA in 94% of reaction mixtures with a false-positive rate of 3% over the course of these experiments (data not shown).

The frequency of explanted splenocytes or peritoneal cells capable of reactivating lytic viral replication was determined by limiting dilution ex vivo reactivation assay. Freshly explanted cells were serially diluted and plated in 96-well microtiter plates seeded with 10^4 wt murine embryonic fibroblasts (MEFs). MEFs and explanted cells were cocultured for 21 days, and viral reactivation was scored by visual inspection for virus-induced cytopathic effect (CPE). To control for possible persistent lytic viral replication in vivo, the extent of preformed lytic virus in explanted cell populations was quantitated by mechanical disruption of parallel cell samples using silica beads prior to plating on indicator MEFs. Such mechanical disruption kills >99% of cells but has minimal effect on infectious virus. Under the infection conditions and at time points used in these experiments, virus persistence in wt and IFN-α/β$R^{-/-}$ mice was at or below the limit of detection of the assay, with an assay sensitivity of ~0.2 PFU per well (89, 91).

All data manipulations and statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Data from limiting dilution viral genome PCR and viral reactivation assays were fitted to a sigmoidal dose-response curve by nonlinear regression to determine the concentration of explanted cells required to achieve 63% viral DNA-positive PCRs or 63% CPE-positive reactivation wells. This cell number was defined according to the Poisson distribution as the reciprocal frequency of viral latency or viral reactivation, respectively (89, 91). The statistical significance of differences observed in latency and reactivation assays was determined by two-tailed, paired *t* test on unmanipulated data.

Detection of lytic virus in organs. At indicated times postinfection, mice were euthanized by halothane inhalation and lung, liver, and spleen were aseptically dissected and homogenized using silica beads in 1 ml of complete Dulbecco's MEM (10% fetal bovine serum). Homogenates were serially diluted in complete Dulbecco's MEM, and infectious virus was quantitated by plaque assay on 3T12 fibroblast monolayers (89). Due to the organ-specific toxicity of lysates on 3T12 monolayers, the limit of detection was 10^1 PFU per lung, 10^3 PFU per liver, and 10^2 PFU per spleen.

RNase protection assay for viral gene expression. Total RNA was prepared from spleens via mechanical disruption using silica beads in the presence of Trizol (Invitrogen, Carlsbad, Calif.). Ten to 20 μg total RNA was hybridized overnight with the γ6 riboprobe set specific for selected γHV68 transcripts (Table 2). A riboprobe specific for mL32 was included as an internal housekeeping signal (35, 66). Two micrograms total RNA from γHV68-infected owl monkey kidney cells was used as a positive control. RNase protection reactions were performed as described elsewhere (66) using the RiboQuant RNase protection assay kit according to the manufacturer's instructions (BD Biosciences, San Jose, Calif.). Resulting protected probes were resolved on a 6% acrylamide denaturing urea gel (40-cm path length), and the signal was visualized using a STORM phosphorimager and quantified with ImageQuant software (GE Healthcare, Chalfont St. Giles, United Kingdom). For the latter, volume measurements with rectangular objects were used to generate phosphorimager volume counts. The statistical significance of differences observed in viral transcript levels was determined by two-tailed, unpaired *t* test on background-subtracted phosphorimager volumes.

RESULTS

IFN-α/β$R^{-/-}$ mice are hypersensitive to γHV68 infection but clear low-dose intranasal infection. Following intranasal infection, 100% of IFN-α/β$R^{-/-}$ mice receiving 1,000 PFU or more succumbed within 12 days postinfection (dpi), whereas

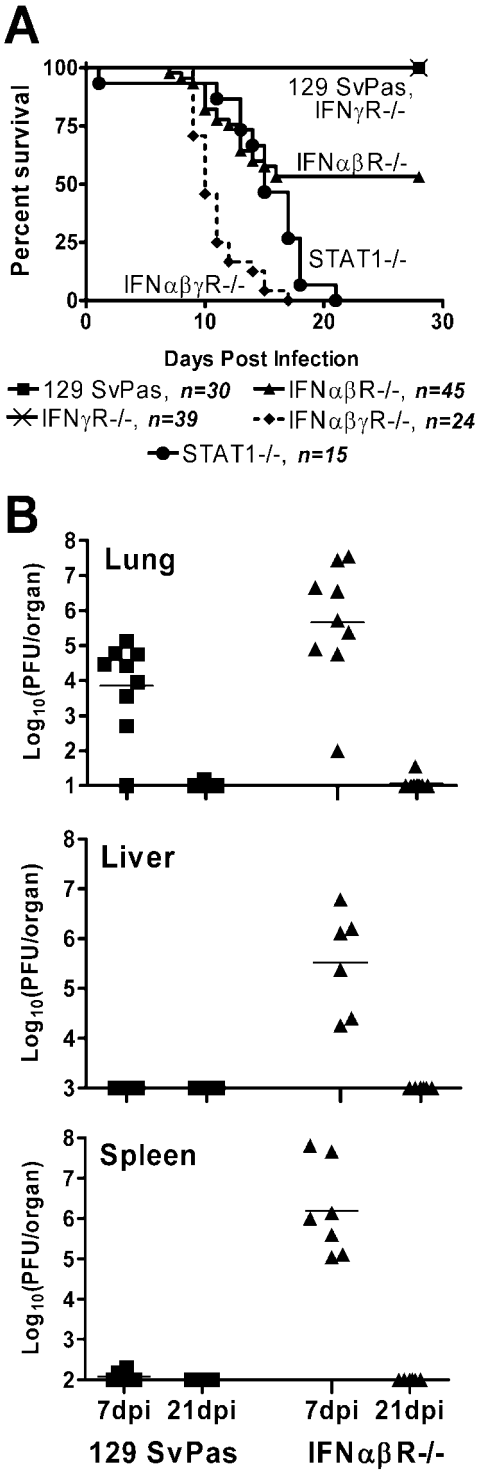

FIG. 1. Lethality and viral replication following γHV68 infection in mice lacking IFN signaling pathways. (A) Age-matched 129SvPas, IFN-α/βR$^{-/-}$, IFN-γR$^{-/-}$, IFN-α/βγR$^{-/-}$, or STAT1$^{-/-}$ mice were infected with 100 PFU γHV68 intranasally and observed daily for mortality. Numbers of mice infected for each genotype are indicated, and results are compiled from three to five independent experiments. (B) Age-matched 129SvPas or IFN-α/βR$^{-/-}$ mice were infected with 100 PFU γHV68 intranasally, and spleen, liver, and lung were harvested 7 to 9 or 21 to 28 dpi. Live virus present in each organ was quantitated by plaque assay. Shown are viral titers present in individual organs from six to nine mice in three independent experiments.

no deaths occurred in wt mice receiving up to 10^6 PFU (data not shown). We determined the kinetics of lethality in mutant mice infected at a dose of 100 PFU intranasally, the approximate 50% lethal dose in IFN-α/βR$^{-/-}$ mice (Fig. 1A). IFN-α/βR$^{-/-}$ mice succumbed to γHV68 during the second week of infection, with no deaths observed after 16 dpi. This is in contrast to IFN-γR$^{-/-}$ mice, which survive acute infection normally but go on to develop fatal large-vessel arteritis (20, 21, 90). Interestingly, we found that mice lacking the key IFN-α/β-induced signaling molecule STAT1 were more sensitive than IFN-α/βR$^{-/-}$ mice, with all STAT1$^{-/-}$ mice infected with greater than 10 PFU succumbing (Fig. 1 and data not shown). Mice lacking receptors for both IFN-α/β and IFN-γ (IFN-α/βγR$^{-/-}$) died more rapidly than either STAT1$^{-/-}$ or IFN-α/βR$^{-/-}$ mice, consistent with the existence of STAT1-independent antiviral effects (32) of IFNs during γHV68 acute infection.

We observed highly elevated lytic virus titers in the lungs of IFN-α/βR$^{-/-}$ mice (Fig. 1B). In addition, while lytic virus was detectable only in the lungs of wt mice, infectious virus was detected in liver and spleen of IFN-α/βR$^{-/-}$ mice by 7 dpi. wt mice typically clear γHV68 within 16 dpi (89), and the kinetics of mortality observed in IFN-α/βR$^{-/-}$ mice (where no deaths were observed after 16 dpi) suggested that these mice had also eliminated acute γHV68 replication. Indeed, by 21 dpi, we were unable to detect live virus in any organ of either genotype (Fig. 1B), indicating that the acute infection had been cleared in all surviving mice. These observations are consistent with those of Dutia et al. (24) and suggest that IFN-α/β is not required for elimination of lytic virus once the cell-mediated immune response has been initiated.

Normal levels of viral latency are established in IFN-α/βR$^{-/-}$ mice. We next determined whether viral latency was established in IFN-α/βR$^{-/-}$ mice that survived the initial infection, using a limiting dilution PCR that determines the frequency of host cells harboring viral genome (Fig. 2) (89, 91). Splenocytes from wt and IFN-α/βR$^{-/-}$ mice harvested 28 dpi displayed equivalent frequencies of latently infected cells, with approximately 1:700 harboring viral genome (Fig. 2A). Peritoneal cells from IFN-α/βR$^{-/-}$ mice displayed a modest increase in the frequency of latently infected cells (1:240 versus 1:620, Fig. 2B). The limiting dilution PCR assay used here can in principle detect phagocytes scavenging viral debris following acute infection. However, if a significant proportion of the cells detected were simply virus-scavenging phagocytes, we would expect much higher frequencies of viral genome in IFN-α/βR$^{-/-}$ mice, given the dramatic increase in lytic replication observed in these mice (Fig. 1B). Since this is not the case, we conclude that the absence of IFN-α/β-induced gene expression does not dramatically alter the absolute frequency of cells that become latently infected following the acute phase of γHV68 infection, suggesting that the level of acute virus replication does not determine the resulting level of viral latency (79).

Peritoneal cells and splenocytes from IFN-α/βR$^{-/-}$ mice display hyperreactivation ex vivo. A hallmark of herpesvirus infection is the periodic reactivation of latent virus to produce lytic viral progeny. The efficiency of viral reactivation ex vivo (and presumably in vivo) can be altered by ablation of either viral (16, 17, 39, 83, 84) or host (10, 15, 28, 30, 41, 43, 44, 50,

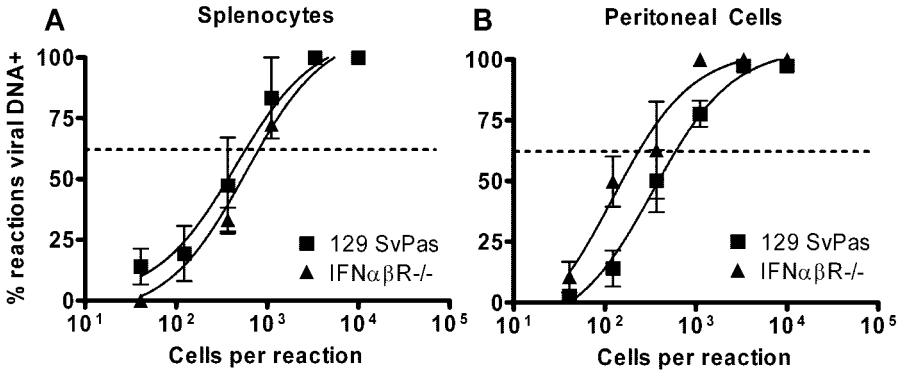

FIG. 2. Establishment of viral latency in wt and IFN-α/β$R^{-/-}$ mice. Splenocytes and peritoneal cells from 129SvPas and IFN-α/β$R^{-/-}$ mice were harvested 28 dpi, and the frequency of viral genome-bearing cells was determined using limiting dilution nested PCR for the viral ORF72 gene. Shown are means and standard errors of the means pooled from three independent experiments. Each sample contained pooled cells from three to five mice. Dashed lines indicate the point of 63% Poisson distribution, determined by nonlinear regression, which was used to calculate the frequency of cells bearing viral genome.

54, 80, 84) genes. To determine whether IFN-α/β regulates viral reactivation efficiency, splenocytes and peritoneal cells from latently infected wt, IFN-α/β$R^{-/-}$, and IFN-γ$R^{-/-}$ mice were explanted 28 dpi and assayed for the capacity to reactivate during culture. At this time point, all three genotypes of mice have cleared high-level lytic infection and harbor nearly identical frequencies of viral genome-positive cells in spleen and peritoneum (Fig. 2) (80). We included samples from IFN-γ$R^{-/-}$ mice as a positive control for IFN effects on virus reactivation, since these mice display chronic low-level virus

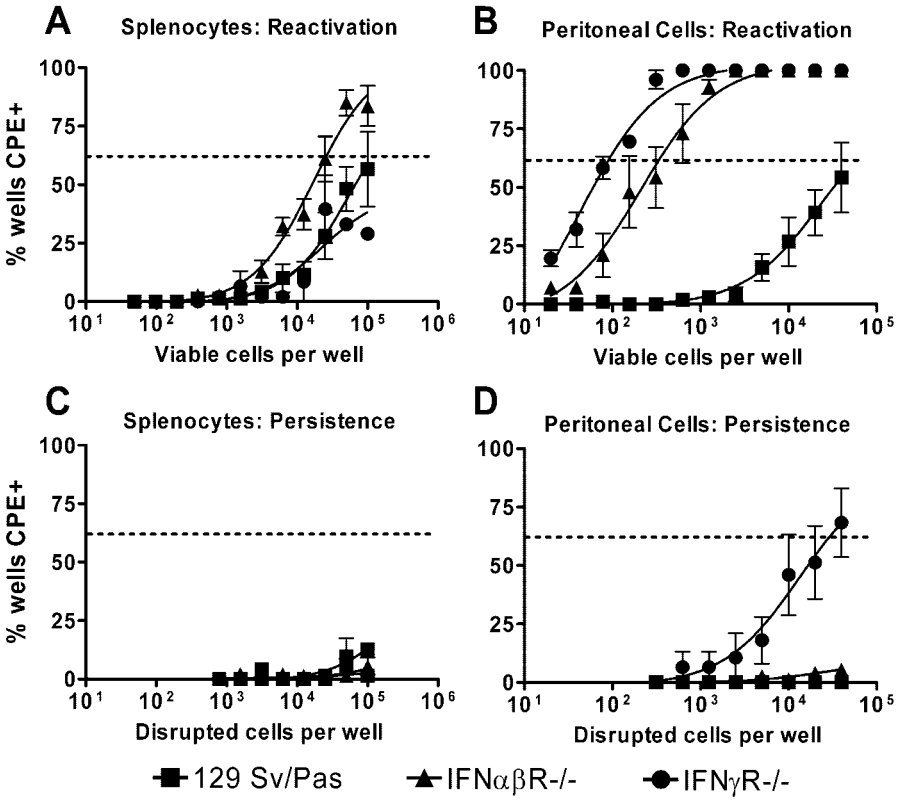

FIG. 3. Reactivation from latency and lytic virus persistence in wt, IFN-α/β$R^{-/-}$, and IFN-γ$R^{-/-}$ mice. Splenocytes (A and C) and peritoneal cells (B and D) from wt, IFN-α/β$R^{-/-}$, and IFN-γ$R^{-/-}$ mice were harvested 28 dpi, and the frequency of cells capable of reactivating lytic virus was determined by 21-day culture on indicator fibroblasts. Wells were scored as positive for viral reactivation based on the presence of complete CPE by microscopic observation (A and B). Parallel samples were mechanically disrupted prior to culture to detect preformed lytic (persistent) virus (C and D). Shown are means and standard errors of the means pooled from three to four independent experiments. Each sample contained pooled cells from three to five mice. Dashed lines indicate the point of 63% Poisson distribution, determined by nonlinear regression, which was used to calculate the frequency of cells reactivating lytic replication.

TABLE 1. Efficiency of viral reactivation

Mouse types fold change, and significance	Frequency of latent viral genome[a]		Frequency of reactivation		Efficiency of reactivation[b]	
	Peritoneal cells	Splenocytes	Peritoneal cells	Splenocytes	Peritoneal cells	Splenocytes
129SvPas	1:620	1:600	<1:40,000	<1:100,000	<0.016	<0.006
IFN-α/βR$^{-/-}$	1:240	1:830	1:360	1:27,000	0.67	0.03
Fold change[c]	2.58	0.72	>111	>3.7	42	5
Significance[d]	0.045	0.082	<0.0001	0.0077		

[a] Viral genome and reactivation frequency data from Fig. 2 and 3 were analyzed by nonlinear regression. The frequency of latency or reactivation was defined as the number of cells per well required to give 63% of samples positive for viral genome by nested PCR or viral reactivation by fibroblast coculture, respectively.
[b] Efficiency of reactivation is defined as the ratio of reactivation frequency to latent frequencies. A reactivation efficiency of 1 would be indicative of reactivation of all latently infected cells during explant culture.
[c] Fold change attributable to effects of IFN-α/βR is defined as the ratio of latency or reactivation frequencies in 129SvPas mice to that observed in IFN-α/βR$^{-/-}$ mice.
[d] Unmanipulated data from latency and reactivation assays were compared using a paired two-tailed *t* test.

persistence in vivo and elevated levels of virus reactivation from peritoneal cells ex vivo (80).

Splenocytes from wt and IFN-γR$^{-/-}$ mice reactivated with low efficiency, with fewer than 1:100,000 cells reactivating lytic replication during the 3-week culture period (Fig. 3A). Reactivation from IFN-α/βR$^{-/-}$ splenocytes was increased at least fourfold above that of wt (~1:27,000). Mechanically disrupted samples from all three genotypes confirmed that no persistent lytic replication was present in the spleen at this time point (Fig. 3C), confirming that true viral reactivation was being measured. These results are consistent with the published lack of effect for IFN-γ in controlling reactivation from splenocytes (80) but indicate that IFN-α/β plays a modest but significant role in reducing reactivation from this tissue.

In contrast, we found that both IFN-α/β and IFN-γ both exert profound but distinct effects in controlling virus reactivation in explanted peritoneal cells (Fig. 3B). IFN-α/βR$^{-/-}$ peritoneal cells reactivated with approximately 100-fold-increased frequency compared to wt cells. When the frequency of reactivation is compared to the frequency of viral genome present in these cell populations (Table 1), it can be demonstrated that the majority of latently infected IFN-α/βR$^{-/-}$ cells reactivate during culture, while fewer than 2% of wt cells reactivate. Such highly efficient reactivation further confirms the hypothesis that the majority of viral genome-positive cells detected in Fig. 2 are in fact latently infected and not simply scavenging viral particles. This high-level reactivation in IFN-α/βR$^{-/-}$ cells occurs in the near-complete absence of detectable persistent viral replication in vivo, as indicated by a 100- to 1,000-fold reduction in detection of CPE in MEFs cultured with equal numbers of mechanically disrupted IFN-α/βR$^{-/-}$ peritoneal cells (compare Fig. 3B and D). In contrast, IFN-γR$^{-/-}$ peritoneal cells displayed highly efficient reactivation accompanied by substantial in vivo persistence (Fig. 3D, compare IFN-α/βR$^{-/-}$ [triangles] to IFN-γR$^{-/-}$ [circles]). Thus, the effects of IFN-α/β during latent γHV68 infection are phenotypically distinct from those of IFN-γ: IFN-α/β is required for control of reactivation but dispensable for control of in vivo persistence, while IFN-γ is required to control both processes.

The effect of IFN-α/β on reactivation wanes at later times postinfection. To determine the duration of IFN-α/β action on viral reactivation, peritoneal cells from wt and IFN-α/βR$^{-/-}$ mice were assayed for reactivation efficiency at 161 dpi (Fig. 4). At these time points, peritoneal cells from the two strains of mice reactivated with equivalent efficiency (wt, ~1:2,100; IFN-α/βR$^{-/-}$, ~1:3,700 [Fig. 4A]). Interestingly, we observed that the extent of viral reactivation in wt mice increased substantially during this time course, from ~1:100,000 at 28 dpi to 1:2,100 at 161 dpi. In contrast, the high-level reactivation in

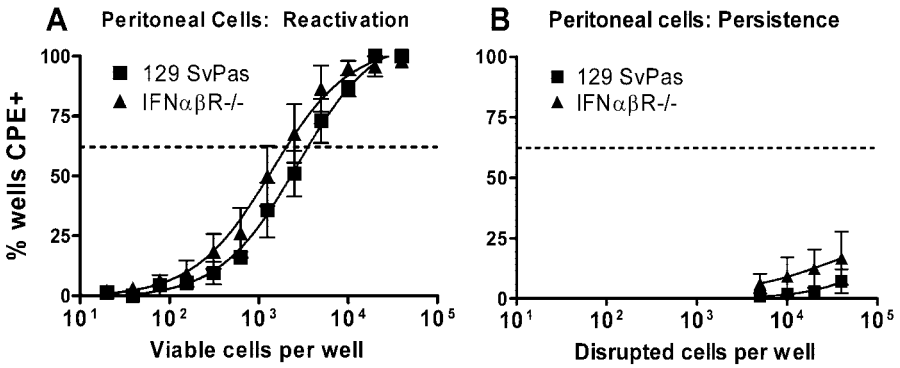

FIG. 4. Effects of IFN-α/βR on viral reactivation wane with time. (A) Peritoneal cells from wt or IFN-α/βR$^{-/-}$ mice were harvested 161 dpi, and the frequency of cells capable of reactivating lytic virus was determined by 21-day culture on indicator fibroblasts as in Fig. 3. (B) Parallel samples were mechanically disrupted prior to culture to detect preformed lytic (persistent) virus. Shown are means and standard errors of the means pooled from four independent experiments. Each sample contained pooled cells from three to five mice. Dashed lines indicate the point of 63% Poisson distribution, determined by nonlinear regression, which was used to calculate the frequency of cells reactivating lytic replication.

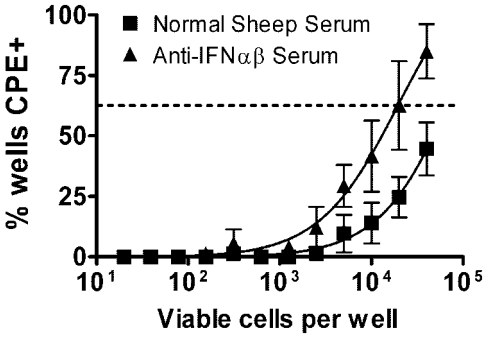

FIG. 5. Depletion of IFN-α/β in vivo results in increased viral reactivation in wt mice. wt mice were infected with 100 PFU γHV68 intranasally. At 21 and 25 dpi, mice received 30 μl control sheep serum or IFN-α/β-depleting sheep serum (5, 33) intraperitoneally in a volume of 500 μl phosphate-buffered saline. Peritoneal cells were harvested 28 dpi, and viral reactivation was assessed on indicator fibroblasts in the presence of a 1:2,500 dilution of control or IFN-α/β-depleting serum. In a parallel experiment, addition of IFN-α/β-depleting serum to reactivation cultures was not sufficient to alter reactivation efficiency in the absence of in vivo depletion (data not shown). Shown are means and standard errors of the means for three individual mice in each group. Dashed lines indicate the point of 63% Poisson distribution, determined by nonlinear regression, which was used to calculate the frequency of cells reactivating lytic replication. The experiment was repeated once with comparable increases in reactivation in IFN-α/β-depleted mice.

IFN-α/β$R^{-/-}$ peritoneal cells decreased approximately 10-fold over time, from 1:360 at 28 dpi to 1:3,700 at 161 dpi. These time-dependent changes in reactivation efficiency were not related to the increased age of the mice in these experiments, since age-matched (24-week-old) wt and IFN-α/β$R^{-/-}$ mice infected for 28 days displayed reactivation efficiencies identical to those in Fig. 3 (data not shown). The level of virus persistence in both genotypes remained very low at both 28 and 161 dpi, with <1% of CPE detected in the reactivation assay

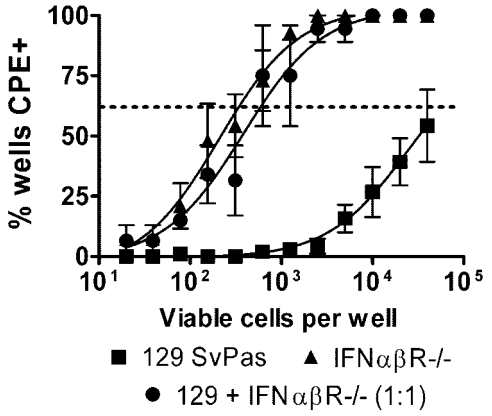

FIG. 6. Effect of IFN-α/β of inhibiting γHV68 reactivation cannot be complemented in *trans*. Peritoneal cells from wt or IFN-α/β$R^{-/-}$ mice were harvested 28 dpi, and the frequency of cells capable of reactivating lytic virus was determined by 21-day culture on indicator fibroblasts as in Fig. 3. Cells from wt and mutant mice were plated independently or mixed in equal numbers prior to serial dilution and plating on indicator fibroblasts. Shown are means and standard errors of the means pooled from two independent experiments. Each sample contained pooled cells from three to five mice.

attributable to preformed virus (compare Fig. 3D and 4B). These data indicate that latency is a dynamic process in both wt and IFN-α/β$R^{-/-}$ mice, with the efficiency of virus reactivation changing over time even in the face of unaltered levels of virus persistence. Furthermore, these data suggest that IFN-α/β functions primarily during the early phase of virus latency and is dispensable during later phases of chronic infection.

IFN-α/β functions to limit virus reactivation in wt mice. Enhanced reactivation from IFN-α/β$R^{-/-}$ cells may reflect defects in the cell-mediated immune response consequent to the high-level lytic replication experienced by these mice early after infection or may reflect developmental defects in the deficient mice that cannot be directly linked to a function for IFN-α/β during latent infection in the wt host. To determine whether IFN-α/β functions during latency in wt mice to limit virus reactivation, we depleted IFN-α/β in vivo following resolution of lytic infection (Fig. 5). wt mice were infected intranasally and starting 21 dpi were depleted of IFN-α/β using a polyclonal sheep serum raised against mouse IFN-α/β (5, 33). At 28 dpi, peritoneal cells from individual mice receiving control or anti-IFN-α/β serum were explanted and assayed for viral reactivation. We found that depletion of IFN-α/β for 7 days resulted in a greater-than-fivefold increase in reactivation from latency (control, ~1:100,000, versus IFN-α/β depleted, 1:19,000; $P = 0.017$). Since wt mice clear γHV68 infection within the first 16 dpi (89), this finding indicates that IFN-α/β continues to exert antiviral effects after lytic infection is cleared and suggests that at least one antiviral effect of IFN-α/β during this period is to limit virus reactivation.

The effect of IFN-α/β on reactivation is dominant and cannot be complemented in *trans*. The alterations in virus reactivation efficiency over time observed in Fig. 4 suggested that reactivation as measured in our ex vivo assay is dependent upon temporally regulated factors acting in the intact host. However, it is possible that some components of the effects of IFN-α/β observed 28 dpi are mediated ex vivo by IFN-α/β or IFN-α/β-induced antiviral factors acting on explanted cells or indicator MEFs. To determine whether explanted wt cells produced soluble factors capable of inhibiting γHV68 reactivation in *trans*, wt and IFN-α/β$R^{-/-}$ peritoneal cells were mixed 1:1 prior to reactivation culture (Fig. 6). wt peritoneal cells were not capable of inhibiting hyperreactivation of IFN-α/β$R^{-/-}$ peritoneal cells during coculture. These data indicate that (i)

TABLE 2. γ6 RNase protection assay riboprobe templates

Predicted protein encoded	Genome coordinates[a]	Protected mRNA size (bases)
K3	25287–24929	360
Rta	68349–68680	330
M8	76044–76343	300
DNA pol	21465–21736	270
v-cyclin	103122–102867	255
gB	18618–18857	240
M2	4331–4110	220
G73	104564–104364	200
G74	105497–105678	180
M11	103628–103786	160
M3	6936–6796	140
M9	94241–94121	120

[a] According to GenBank accession no. U97553.

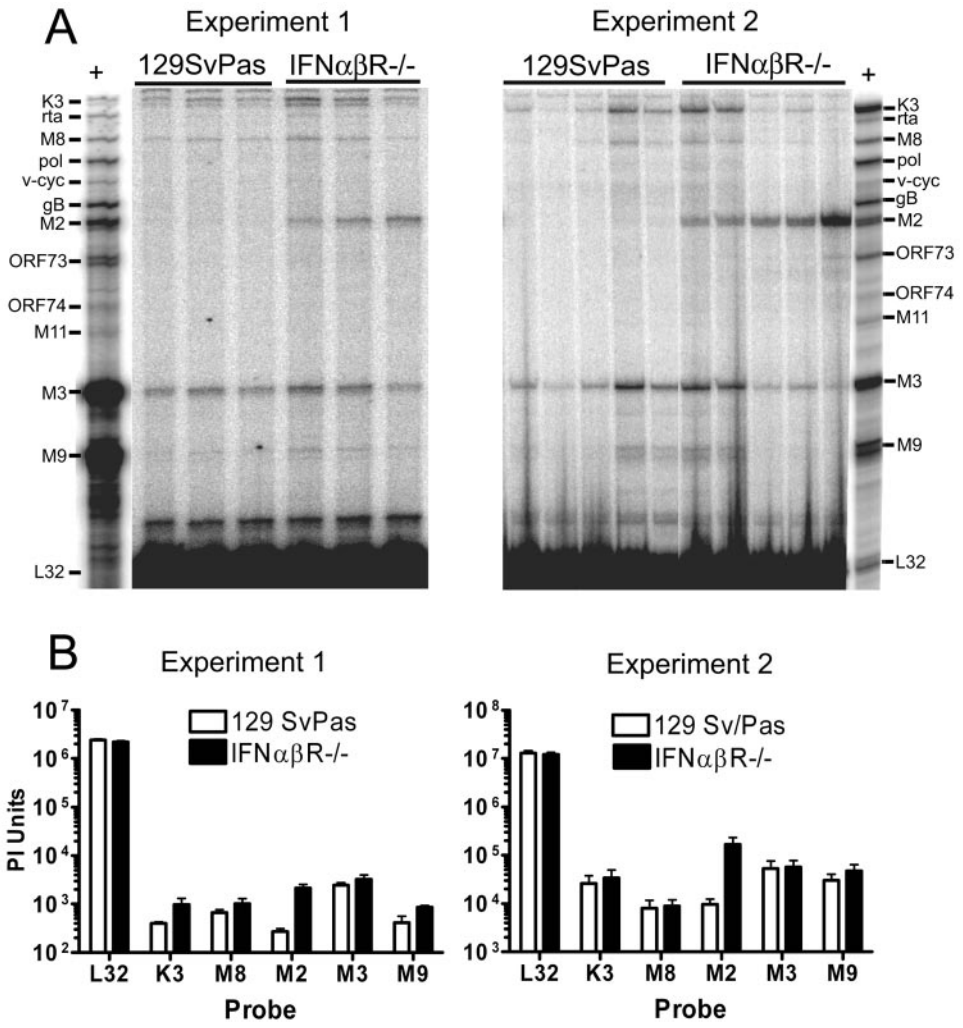

FIG. 7. Latent viral gene expression in wt and IFN-α/βR$^{-/-}$ mice. Spleens were harvested from wt or IFN-α/βR$^{-/-}$ mice 28 dpi, and total RNA was purified. (A) Twenty micrograms of total RNA was hybridized overnight with the γHV68-specific γ6 riboprobe set and digested with RNase, and protected fragments were resolved by polyacrylamide gel electrophoresis. Each lane represents pooled spleen RNA from two to three mice (experiment 1) or from individual mice (experiment 2). Two micrograms of total RNA from γHV68-infected owl monkey kidney cells was used as a positive control (+). Shown are phosphorimages from two independent experiments. (B) Viral and cellular transcript levels were quantified by phosphorimager analysis and are presented as phosphorimager (PI) units. The means and standard errors of the means for phosphorimager units are shown for each transcript following quantitation of phosphorimages in panel A.

wt peritoneal cells do not produce non-IFN-α/β *trans*-acting factors that can dominantly inhibit viral reactivation during ex vivo culture and (ii) any IFN-α/β produced during the ex vivo assay is not sufficient to inhibit CPE on indicator MEFs. Consistent with this observation, we have found that the frequency of reactivation observed from wt peritoneal cells is identical when measured using wt MEFs or MEFs lacking the IFN-α/β and IFN-γ receptors, demonstrating that IFNs function in vivo to alter the inherent reactivation potential of explanted cells (75a).

Expression of viral latency-associated gene M2 is upregulated in IFN-α/βR$^{-/-}$ mice. Our findings are consistent with the hypothesis that the reactivation efficiency of explanted cells, as measured in this assay, is intrinsic to the cells at the time of explant and is modulated in vivo by host factors including IFN-α/β. Such modulation of viral reactivation could be mediated indirectly, through modulation of the adaptive immune response against γHV68-infected cells, or by direct alteration of viral gene expression during latency. To determine whether IFN-α/β-dependent alterations in viral gene expression could be observed in vivo during viral latency, we employed an RNase protection assay that detects expression of 12 lytic and latent-phase viral transcripts in cells directly harvested from infected mice (Table 2) (66) (Fig. 7). RNA from lytically infected owl monkey kidney cells, which contains detectable levels of all viral transcripts, was used as a positive control. Expression of viral lytic genes (including gB and pol) was not detected in splenocytes of either genotype of mice, consistent with the absence of lytic viral replication in this compartment (Fig. 1B and 3B). However, viral latency-associated transcripts, including K3, M3, M9, and M8, were detected in splenocytes from both wt and IFN-α/βR$^{-/-}$ mice at equiv-

alent levels ($P > 0.05$ for all comparisons). This overall pattern of viral transcripts observed in both wt and IFN-α/βR$^{-/-}$ mice is consistent with elimination of lytic infection and establishment of a latent gene expression pattern (2, 25, 53, 66, 86). In contrast, the viral latency-associated M2 transcript was upregulated 5- to 20-fold in splenocytes from IFN-α/βR$^{-/-}$ mice (Fig. 7B, $P = 0.01$ [experiment 1], $P = 0.041$ [experiment 2]). Thus, lack of IFN-α/β-induced signaling events during viral latency correlates with specific alterations in expression of viral latency-associated transcripts, suggesting a possible mechanism for IFN-α/β inhibition of viral reactivation.

DISCUSSION

In this report, we demonstrate an important antiviral role for IFN-α/β that is evident after the adaptive immune response is established and acute infection has been cleared. It was previously reported that γHV68 latency in splenocytes from IFN-α/βR$^{-/-}$ mice was slightly elevated at early times after infection but returned to wild-type levels by 21 dpi (24). Based on this finding, the authors concluded that IFN-α/β plays a critical role during lytic γHV68 infection but is not required for normal regulation of latency. We and others have found, however, that host immune response genes are differentially required for the control of latency in distinct anatomic compartments, likely reflecting cell-type-specific mechanisms of pathogen resistance. For example, IFN-γ is required for elimination of persistent viral replication in peritoneal cells and large vessels but is dispensable for control of viral replication and latency in the spleen (Fig. 3) (20, 21, 80). We therefore revisited the role of IFN-α/β in regulating γHV68 latency and assessed viral latent infection in both splenocytes and peritoneal cells and quantitated the efficiency of viral reactivation using sensitive assays for latent viral genome and viral reactivation. Our results are consistent with those of Dutia et al. (24), in that a modest but significant effect of IFN-α/β was observed on reactivation of splenocytes. However, we significantly extend this observation and demonstrate here (i) a critical and temporally regulated role for IFN-α/β in inhibiting γHV68 reactivation from peritoneal cells, (ii) the continued function of an IFN-α/β-induced antiviral pathway(s) that regulates viral latency in wild-type mice, and (iii) a specific effect of IFN-α/β on latent viral gene expression patterns in vivo.

Distinct IFN-α/β and IFN-γ functions during γHV68 acute infection. The differences in virus-induced lethality during acute infection in wt, IFN-α/βR$^{-/-}$, IFN-γR$^{-/-}$, IFN-α/βγR$^{-/-}$, and STAT1$^{-/-}$ mice in Fig. 1 demonstrate functional diversity of IFNs during γHV68 infection. Not surprisingly, IFN-α/β plays a critical role in restraining initial virus replication, spread, and lethality. Although IFN-γ is not required for normal control of acute γHV68 infection (Fig. 1) (70, 80, 90), the uniform lethality of infection in IFN-α/βγR$^{-/-}$ mice indicates that the IFN-γ receptor performs antiviral functions during the acute phase of infection when the IFN-α/β receptor is absent. In addition, since IFN-α/βγR$^{-/-}$ mice succumb more rapidly than STAT1$^{-/-}$ mice, STAT1-independent antiviral functions for IFN-α/β and/or IFN-γ must be operant during acute γHV68 infection, a noncanonical pathway for IFN action which is also important during murine cytomegalovirus and Sindbis virus infection (32). However, STAT1-dependent pathways appear to protect IFN-α/βR$^{-/-}$ mice from lethality during the second week of infection, since 50% of IFN-α/βR$^{-/-}$ mice survive past this point and STAT1$^{-/-}$ mice uniformly succumb. These data are consistent with a dominant role for IFN-α/β-induced, STAT1-dependent genes in restraining acute γHV68 replication, with IFN-γ-dependent and STAT1-independent pathways performing auxiliary or perhaps redundant antiviral functions.

Distinct IFN-α/β and IFN-γ functions during γHV68 latent infection and reactivation. We found that the mechanisms utilized by IFN-α/β and IFN-γ in controlling γHV68 chronic infection are functionally distinct. First, we did not observe chronic persistent viral replication in peritoneal cells of IFN-α/βR$^{-/-}$ mice, while cells from IFN-γR$^{-/-}$ mice contained preformed lytic virus (Fig. 3D) (80). Second, we did not observe large-vessel arteritis or concomitant delayed lethality in IFN-α/βR$^{-/-}$ mice, pathological hallmarks of γHV68 infection in mice lacking IFN-γ responsiveness (data not shown and reference 90). However, IFN-α/β and IFN-γ display similar tissue-specific effects in modulating reactivation, with both pathways critical for restraining reactivation of peritoneal cells while exerting less of an effect in splenocytes (Fig. 3) (80). This observation suggests that discrete gene expression pathways control γHV68 persistence in peritoneal cells, with IFN-α/β-induced pathways regulating reactivation but not persistence, while IFN-γ-induced pathways are critical to regulating both viral processes. Comparison of host gene expression patterns during γHV68 latency in wt, IFN-α/βR$^{-/-}$, and IFN-γR$^{-/-}$ mice may elucidate the IFN-induced host genes performing these distinct functions during latent infection.

Despite the high-level systemic viral replication observed in IFN-α/βR$^{-/-}$ mice during acute infection, the frequency of latently infected cells in these mice is essentially the same as that observed in wt mice within 1 week after viral clearance (Fig. 2). The ratio of viral genome-positive cells to reactivation events is nearly 1 in IFN-α/βR$^{-/-}$ peritoneal cells despite the nearly complete absence of preformed infectious virus (Table 1), a finding which is most compatible with the hypothesis that γHV68 infection is latent in vivo but that every virus-infected cell is capable of reactivation when explanted (74, 87). We therefore favor the interpretation that (i) the viral genome detected in Fig. 2 resides in a reactivatable, latently infected compartment and not simply in lytically infected cells or phagocytes clearing viral debris and (ii) the set point for the frequency of latently infected cells is not dependent upon IFN-α/β and is dissociated from the level of acute viral replication. This observation is consistent with previous work from our group and others, which demonstrates equivalent establishment of latency in mice infected with a 6-log range of virus inoculum, strongly supporting a physiologic uncoupling of the processes that regulate the level of acute and latent infection (27, 79).

IFN-α/β induction and mechanisms of action during viral latency. As with most experimental herpesvirus models, reactivation of γHV68 is difficult to detect in the intact host, necessitating the use of aggressive immunosuppression or explantation of latently infected cells to trigger reactivation. This likely reflects the redundant effects of the host T- and B-cell response to infection in eliminating reactivating cells early in the reactivation process and in neutralizing lytic progeny produced by cells that complete the viral life cycle (30, 40, 43, 54).

In the γHV68 system, viral genome-bearing cells isolated from wt mice after the establishment of latency reactivate inefficiently when explanted (Table 1). Our data indicate that the IFN-α/β system plays an active role in this process of generating cells that inefficiently reactivate when explanted. While our results clearly indicate that IFN-α/β is important for regulating the efficiency with which cells reactivate ex vivo, it is less clear how to relate this observation to events inhibiting viral reactivation in vivo. Since we initially employed an ex vivo reactivation assay and genetically deficient mice to detect these effects of IFN-α/β on reactivation, we considered it critical to complement this result with direct in vivo analyses.

We found that IFN-α/β continues to be produced following resolution of acute infection in wt mice and that depletion of IFN-α/β in vivo leads to increased viral reactivation in explanted cells. In addition, the inability of wt cells to inhibit reactivation from cocultured IFN-α/βR$^{-/-}$ cells (Fig. 6) argues that the low efficiency of reactivation from wt cells is intrinsic to these cells and is determined by processes occurring in the host, not by artifacts of the ex vivo assay. This hypothesis is further strengthened by the time-dependent changes in reactivation efficiency observed in Fig. 4, which clearly demonstrate that temporally regulated in vivo factors control ex vivo reactivation efficiency. Finally, with the demonstration of specific alterations in viral transcription in the absence of IFN-α/β, we provide molecular evidence for in vivo effects of IFN-α/β on viral gene expression (Fig. 7). These independent lines of evidence indicate that IFN-α/β functions in vivo to regulate viral latency. It is important to underscore the fact that our data do not directly link IFN-α/β to increased viral reactivation in the infected mouse. However, since the in vivo function of IFN-α/β during latency is associated with a profound increase in viral reactivation efficiency ex vivo, this assay provides a tractable system for dissection of IFN-α/β function in the host.

The observed effects of IFN-α/β on latently infected cells could be mediated at several different levels in vivo. Inhibition of γHV68 reactivation may result from direct stimulation of IFN signaling pathways in latently infected cells, resulting in modulation of cellular or viral transcription to create an intracellular state that represses viral reactivation. IFN-α/β is basally expressed at low levels in the absence of known infections, and this basal expression is required for a robust IFN response following viral challenge (78). Belardelli et al. noted that explanted peritoneal macrophages are innately resistant to virus infection and that this resistance is overcome by neutralizing basal IFN-α/β expression in vivo (5). Since >90% of the peritoneal cells that harbor the γHV68 genome during latency are macrophages (92), our results in Fig. 5 suggest that the innate resistance of peritoneal macrophages to viral replication may also function to restrain reactivation of latent virus infection. Consistent with this hypothesis, IFN-α/βR$^{-/-}$ primary macrophages have functional defects in response to lipopolysaccharide treatment, including failure to upregulate inducible nitric oxide synthase, indicating that IFN-α/β acts on macrophages in an autocrine feedback loop to activate antimicrobial gene expression pathways (38). We hypothesize that constitutive IFN-α/β stimulation of latently infected cells may also regulate viral gene expression patterns in latently infected cells to restrict viral reactivation.

The specific targeting of the γHV68 M2 transcript by IFN-α/β is consistent with this hypothesis, since the M2 gene is required for efficient viral reactivation from splenocytes (39). Of particular interest for our study is the recent observation that the M2 protein can inhibit STAT-dependent interferon signaling, suggesting that M2 may be both an inhibitor of IFN action and a target of IFN antiviral effects (49). Indeed, given the profound effects of IFN-α/β that we observe in IFN-α/βR$^{-/-}$ mice, M2 may function during reactivation of latently infected cells to inhibit the effects of IFN-α/β on this process. Interestingly, although latent viral genes were easily detectable in splenic RNA, we have been unable to detect abundant expression of viral genes in peritoneal cells, despite the relatively high efficiency of viral reactivation from peritoneal cells (Table 1 and data not shown). Since the cell types infected in each anatomic site differ, with the majority of virus in the peritoneum present in macrophages while most splenic viral genome resides in B cells, this suggests that cell-type-specific latent gene expression patterns exist in vivo. However, it is unlikely that M2 is the sole target of IFN-α/β effects on viral gene expression during latency, and a global comparison of γHV68 transcription patterns in the presence and absence of IFN signaling will be required to fully dissect the interactions between host IFNs and latent viral gene expression.

Alternatively, it is possible that the effects of IFN-α/β and γHV68 reactivation are not mediated directly at the level of IFN signaling in the latently infected cell and may instead be the indirect result of IFN-α/β effects on the virus-specific immune response. Our observation that depletion of IFN-α/β from wt mice from 21 to 28 dpi results in increased reactivation efficiency in explanted cells (Fig. 5) seems inconsistent with this hypothesis, since in these mice the antigen-specific immune response has already matured to the extent that acute infection has been eliminated (Fig. 1B). However, IFN-α/β depletion resulted in only a partial enhancement of γHV68 reactivation compared to IFN-α/βR$^{-/-}$ mice, suggesting that IFN-α/β-dependent immune modulation during acute infection may be required for complete inhibition of virus reactivation from latency. As a precedent for such a mechanism, high-level replication of lymphocytic choriomeningitis virus in the absence of IFNs may lead to T-cell exhaustion and establishment of persistent lytic viral replication (62). In addition, the induction of IFN-α/β in response to Toll-like receptor ligation promotes an efficient CD8$^+$ T-cell response (36, 37, 52). Given the critical role of T lymphocytes in controlling γHV68 latency (8, 54, 71, 73, 81) and the capacity of IFN-α/β to prolong effector T-cell viability during the activation phase (52), it is possible that prolonged maintenance of an effective γHV68-specific CTL response may require chronic IFN-α/β expression during viral latency.

Temporal regulation of viral reactivation efficiency: latency as prolonged, dynamic inflammation. We found that viral reactivation efficiency is temporally regulated during latency. Early during latency (28 dpi), IFN-α/β-induced pathways are critical for inhibiting reactivation. Over time, however, the IFN-α/β pathway becomes dispensable, with wt and IFN-α/βR$^{-/-}$ mice displaying equivalent reactivation efficiency at 161 dpi. During this same time period, the level of preformed infectious virus (indicative of either viral persistence or in vivo reactivation) remains near the limit of detection in both wt and

IFN-α/βR$^{-/-}$ mice. Interestingly, we found that peritoneal cells from wt mice substantially increase reactivation efficiency during the first 5 months of infection, suggesting that host pathways inhibiting reactivation gradually wane for the first several months of latent infection. This finding is consistent with prolonged but gradually declining T-cell activation and proliferation observed in γHV68-infected mice and suggests that early viral latency is a period of active inflammatory resistance against latent virus reactivation (29, 34, 76, 77). Our findings suggest that IFN-α/β-induced pathways play an important role in restraining viral reactivation during this time period.

IFN-α/β and evolution of viral latency. Mounting genetic and biochemical evidence indicates that both the lytic and latent genetic programs of herpesviruses have evolved under selective pressure from the host IFN-α/β system. Genetic analysis of the human gammaherpesviruses EBV and KSHV suggests a dynamic relationship between the latent viral gene expression program and cellular IFN-α/β signaling pathways. In order to permit reactivation and virus replication, KSHV encodes two proteins—rta and orf45—which degrade and inhibit cellular IFN response factor 7 (IRF-7), a key signaling molecule in the induction of IFN-α/β antiviral responses (37, 95, 101). KSHV encodes three viral IRFs (vIRFs), homologs of cellular IFN-induced and activated transcription factors, which are capable of inhibiting signaling induced by IFN-α/β (4, 94). A subset of these vIRFs are expressed during viral latency, suggesting that IFN-α/β exerts antiviral effects on latent cells, thereby leading to selective pressure to maintain vIRF expression (9, 31, 65, 102). Surprisingly, vIRF3 stimulates the transcriptional induction of the cellular IRF-7 (51), and KSHV maintains sequences in at least one key lytic promoter that binds cellular IRF-7, resulting in the inhibition of viral replication in the presence of an IFN-α/β response (88). In addition, the promoter for the KSHV viral interleukin 6 (vIL-6) gene is transactivated by IFN-α/β, and its upregulation restores IL-6 autocrine growth signals lost when IFN-α/β downregulates cellular IL-6 (13, 63).

Recent evidence indicates that EBV proteins expressed during latency also trigger the activation of at least a subset of the IFN-α/β response genes in a manner which may promote survival of the latently infected cell (96). Among the genes induced by EBV latency-associated proteins is IRF-7, which binds to interferon-stimulated response elements in the viral genome to stimulate transcription of viral latency proteins (61, 97, 98). The existence of multiple mechanisms to either inhibit or subvert IFN-α/β responses in the human gammaherpesviruses indicates that these viruses have evolved under pressure from the IFN-α/β system. Left open is the question of whether the effects of IFN-α/β shown are deleterious to virus or represent a viral strategy to usurp ongoing host cytokine responses and promote stable lifelong latent infection.

Taken together with genetic data from the human gammaherpesviruses, our observations point to a significant evolutionary selective pressure exerted by IFN-α/β on gammaherpesvirus latent gene expression patterns and indicate that this virus family has adapted mechanisms that both inhibit IFN-α/β action and incorporate IFN-α/β signaling molecules into viral transcriptional regulatory pathways. An obvious inference from this genetic evidence is the hypothesis that host IFN-α/β antiviral effects are not restricted to the innate immune response but continue following the resolution of acute infection. Our results support this hypothesis and provide a system to dissect the interplay between viral latent gene expression, viral reactivation, and the host IFN-α/β system during latent infection in the intact natural host.

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